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ORIGINAL ARTICLE

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# Differential sensitivity to etoposide (VP-16)-induced S phase delay in a panel of small-cell lung carcinoma cell lines with G1/S phase checkpoint dysfunction

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**Abstract** *Purpose*: The highly schedule-dependent cytotoxic agent etoposide (VP-16) is initially effective in the treatment of small-cell lung cancer (SCLC), particularly in multidrug combination chemotherapy. Heterogeneity in cellular sensitivity to cell cycle arrest may underpin the inadequacy of low-dose extended-cycle single-agent regimes in tumours with partially dysfunctional apoptotic signalling pathways. We have studied the longevity and dose dependency of cell cycle and to a limited extent the apoptotic responses of a panel of seven unselected SCLC cell lines, screened for *TP53* status. *Methods*: Cells were analysed using flow cytometry for the cell cycle responses and field inversion gel electrophoresis for apoptotic patterns. The mitotic inhibitor nocodazole was used to assess and correct cell line response data for differences in cell cycle traverse per se. *Results*: An overall lack of G1/S arrest and muted DNA fragmentation were consistent with the presence of *TP53* gene abnormalities. Maximal G2 arrest but with clear recovery potential occurred at an exposure dose (ED, concentration of drug x time) value of approximately 24 μM·h. Higher doses (ED values >48 μM·h) revealed a wide variation in S phase delay that was independent of population doubling time and could not be compensated for by drug concentration changes alone. *Conclusion*: The results suggest that heterogeneity in the in vitro sensitivity of unselected SCLC cell lines to S phase arrest is demonstrable at ED values projected to be critical for clinical activity. Such variation in S phase responsiveness may reflect differences in checkpoint activation and offer a functional target for the design of more-effective combination therapy.

**Key words** VP-16–213 · DNA topoisomerase II · Cell cycle · *TP53* tumour suppressor gene · DNA fragmentation

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## Introduction

Small-cell lung cancer (SCLC) typically shows initial sensitivity to chemotherapy with responsiveness to the DNA topoisomerase II inhibitor etoposide (VP-16), although long-term treatment often fails. Attempts to use oral etoposide in extended treatment cycles have also revealed under-performance compared with standard chemotherapy [7, 30]. The reasons for failure are not clear, but must fundamentally involve the inability to attain critical thresholds of active agent sufficient to contend with inter- and intra-tumour heterogeneity in cellular responsiveness. General pathways for resistance to the antiproliferative and cell killing effects of DNA damage may involve the presence of a mutant or dysfunctional p53 phenotype [2, 17, 19], given that *TP53* mutations or abnormalities are found in around 70% of SCLC cases [8]. Together with the loss of RB gene function [15], the implication is that SCLC tumours may lack normal cell cycle control when exposed to DNA damaging agents [25]. Several other factors can effect treatment failure, including modified drug availability through multidrug resistance pathways and drug target-related factors, such as changes in the target enzyme topoisomerase II [6, 10, 28]. The current study addresses the heterogeneity in VP-16-induced cell cycle perturbation for a panel of SCLC cell lines and attempts to relate the thresholds for cycle arrest with published clinical experience.

DNA damage induced by cytotoxic topoisomerase II inhibitors such as VP-16 is a consequence of the inter-

ruption of the enzyme catalytic cycle [18, 27]. Typically, expression of this target enzyme is high in lung tumours [9]. The expression of the 170-kilodalton form, topoisomerase IIα, is cell cycle regulated and enzyme levels may also increase as SCLC cells experience VP-16-induced late cell cycle delay [28]. VP-16 acts within the DNA-enzyme complex to stabilise a covalent intermediate, termed the 'cleavable complex' [3], initiating a sequence of events leading to cell death. Exposure to topoisomerase II poisons induces a prominent G2 delay [4, 28], particularly in p53 dysfunctional cells, with evidence that S phase progression may also be affected at higher dose levels [28].

We suggest that the effectiveness of early phase treatment of SCLC will depend upon the operation of factors that abrogate cell cycle arrest and cell death. Central to this issue is an appreciation of the spectrum of cell cycle arrest and cell death responses shown by SCLC cells when challenged with agents such as VP-16. Here we have studied the cell cycle arrest and DNA fragmentation patterns for a panel of non-selected SCLC cell lines screened for their *TP53* status, while accounting for the confounding factor of differences in cell cycle traverse rates. The results reveal a wide range of sensitivities to pre-G2 cycle delay for the SCLC cell lines that may underpin initial responsiveness at clinically effective drug dose levels, this feature potentially representing a limiting factor in the evasion of drug action.

## Materials and methods

### Cell lines, cell culture, drugs and MTT assay

The human follicular B-lymphoma cell line DoHH2 was derived by Dr. J.C.Kluin-Nelemans (Leiden, The Netherlands) [16] and expresses wild type p53. DoHH2 was routinely maintained in RPMI 1640 supplemented with 5% fetal calf serum supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin and 2 mM glutamine. NCI-H69, a human SCLC cell line, was obtained from Dr. P. Twentyman [1]. The SCLC cell lines described in this study with COR designations were early passage lines derived from clinical specimens by Dr. P. Twentyman as described by Baillie-Johnson et al. [1]. Cell culture and mutation analyses on the SCLC panel for exons 4–8 in the *TP53* gene have been described previously ([29]; P Rabbitts, personal communication). VP-16 (VP-16–213, Vepesid; molecular weight 588) was provided as a 34 mM stock solution (Bristol Meyers Pharmaceuticals, Syracuse, N.Y., USA) and stored at 4 °C. Nocodazole (Sigma) was dissolved as a 10 mM solution in dimethyl sulfoxide and stored at 4 °C. Growth inhibition experiments were performed using the 3–4, 5 dimethylthiazol-2, 5 diphenyltetrazolium bromide (MTT) assay as described previously [31] for continuous exposure to VP-16 for 5 days.

### Cell cycle analysis

Cells were ethidium bromide stained for DNA content as described previously [5] for the flow cytometric analysis of cell cycle distribution [32]. For reference, the previously published [29] mean values for the percentages of cells in G1, S phase and G2/M, respectively for control cultures were as follows: 42.1, 38.3 and 19.6 (NCI-H69); 51.1, 32.6 and 16.8 (COR-L32B); 56.1, 26.0 and 17.8 (COR-L47F); 44.3, 42.3 and 13.5 (COR-L51B); 35.3, 37.6 and 27.0 (COR-L96C); 48.1, 37.8, and 14.2 (COR-L103); 44.9, 35.2 and 20 (COR-L266B); 50.5, 31.2 and 18.3 (COR-L279).

### DNA fragmentation detection by field inversion gel electrophoresis

Embedded cells in 0.8% agarose were lysed overnight in 0.2 μg/ml proteinase K solution (100 mM EDTA pH 8.0, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine), in a 50 °C water bath. After washing in 20 mM TRIS pH 8.8, 50 mM EDTA, plugs were kept at 4 °C. The embedded cellular DNA was loaded onto a 1% agarose gel (0.5% TBE) and run in a field inversion gel electrophoresis unit (Bio-Rad FIGE Mapper) at 15 °C. Two programs were used to separate DNA fragments of 1–50 kilobases (kb) or 50–1,000 kb. Fragments were quantified by UV ethidium bromide densitometry with reference to DNA size markers.

## Results

### Characteristics of SCLC cell lines

Most of the SCLC cell lines, including NCI-H69, had DNA contents similar to that of peripheral blood lymphocytes (Table 1). The exception was COR-L47F (no detectable mutations in exons 4–8 of *TP53*), which was clearly hypo-diploid. COR-L96C (35%) presented the lowest and COR-L47F (56%) the highest percentage of cells in G1 [29]. An additional SCLC cell line [29], COR-L88B, was screened and found to comprise two populations with different DNA content and was therefore not suitable for the VP-16 cell cycle arrest study. Population doubling times (Td) for the COR SCLC lines were in the range of 86 to $>240$ h (Table 1), consistent with other reports of slow growth rates [10].

A previous study [29] has revealed *TP53* tumour suppressor gene changes, and the cell lines are grouped accordingly in Table 1. COR-L103 cells displayed a *TP53* exon 7 alteration, while COR-L51B and COR-L279 cells exhibited *TP53* exon 5 mutations. In NCI-H69 and COR-L266B cells, *TP53* sequencing showed stop codons in exons 5 and 8 respectively, the loss of protein being confirmed by parallel enzyme-linked immunosorbent assays (ELISAs) (SS; data not shown). A *TP53* mutation in COR-L32B was predicted to produce no amino acid change and therefore offers no clear evidence of *TP53* gene abnormalities. The COR-L32B cell line was included in the VP-16 cell cycle study, although it offers no unequivocal evidence of a *TP53* gene abnormality. COR-L47F showed no mutations in exons 5–8. Further gene characterisation and VP-16 cell cycle studies were not pursued for COR-L47F due to its grossly abnormal DNA content. There were no detectable mutations in exons 4–8 of *TP53* in COR-L96 C. However, in the case of COR-L96 C there is a suggestion of p53 alteration since both immunocytochemistry and ELISA indicate stabilisation of the protein (PR and SS; data not shown).

Difficulties in handling microscale cultures of cells prone to aggregation resulted in some variation of MTT assay results. The mean $IC_{50}$ values for the COR cell

lines ranged from 32 to 230 nM VP-16 and were generally more sensitive than NCI-H69. MTT assay results varied most for COR-L96C, although the results obtained suggest an $IC_{50}$ value of $< 100$ nM.

## VP-16-induced DNA fragmentation

To evaluate the kinetics of cell death in drug-treated representative NCI-H69 cultures, VP-16-induced DNA fragmentation was measured using the sensitive method of field inversion gel electrophoresis to identify the generation of low (5–50 kb) or high (50–700 kb) molecular size DNA fragments ([20]; Table 2). In NCI-H69 control cultures, levels of induced fragmentation appeared to occur equally for the two size ranges and there were only moderate changes in fragmentation rates after 24 and 48 h exposure, even when the VP-16 concentration was increased from 0.25 to 2 μM. Elevated DNA fragmentation was apparent even after cell washing and re-incubation in fresh medium for 24 h, although after a 72-h recovery period no elevated fragmentation could be detected. The MTT and fragmentation data provide a dose range (up to 2 μM) and time frame (24–48 h) for the monitoring of biologically relevant events in the cell cycle study.

## VP-16-induced cell cycle perturbations in SCLC cell lines

To permit the detection of general responses, Fig. 1 shows the group means for seven SCLC lines for the four exposure/recovery conditions used in Table 2. In this initial screen, all lines were grown as resuspension cultures [29] to maximise response potential and all cells with G1-G2 range DNA contents, irrespective of nuclear light scatter characteristics, were analysed.

The data (Fig. 1a–c) indicate a significant dose-dependent reduction in G1 fraction at 24 and 48 h exposure, suggesting relatively unimpaired exit from G1 but reduced G1 entry. The G1 emptying is correlated with a delay of cells in G2/M that diminishes as the VP-16 concentration increases beyond 0.5 μM and takes up to 48 h to develop. The results for the SCLC panel are consistent with previous reports of schedule dependency [21, 24, 26] and a two-step G2 accumulation for leukaemic cells [4, 14]. The release of cells from VP-16

**Table 1** Summary of characteristics of small-cell lung carcinoma cell lines (*ND* not determined)

| Cell line | DNA content[a] (±SE) | Td[b] (h ± SE) | VP-16 $IC_{50}$[c] (nM; ± SE) |
|---|---|---|---|
| Homozygous *TP53* mutation or deletion | | | |
| NCI-H69 | 0.98 | 58 ± 10 | 232 ± 67 |
| COR-L266B | 1.02 ± 0.01 | 149 ± 24 | 230 ± 54 |
| COR-L279 | 1.05 ± 0.02 | 96 ± 14 | 95 ± 70 |
| Heterozygous *TP53* mutation | | | |
| COR-L51B | 1.21 ± 0.02 | 118 ± 34 | 32 ± 21 |
| COR-L103 | 0.93 ± 0.01 | 202 ± 43 | 221 ± 139 |
| *TP53* mutation with no amino acid change | | | |
| COR-L32B | 0.95 ± 0.02 | > 240 | 63 ± 45 |
| No mutations in exons 4–8 | | | |
| COR-L96C | 0.89 ± 0.02 | 86 ± 7 | ND |
| No mutations in exons 5–8 | | | |
| COR-L47F | 0.60 ± 0.02 | 103 ± 17 | 56 ± 18 |

[a] Determination of mean value (±SE) by flow cytometry relative to a human peripheral blood lymphocyte standard (DNA assumed to be 8.3 pg/cell)
[b] Td, population doubling time determined by growth curve analysis
[c] $IC_{50}$ is VP-16 concentration required to reduce cell density to 50% of control density over a 5-day incubation period
[d] DNA content of the lower DNA content subpopulation (> 90%)

**Table 2** Field inversion gel electrophoresis of DNA fragmentation in NCI-H69 cells during VP-16 exposure and culture recovery

| Condition | Recovery | | Relative increase in fragmentation at VP-16 concentration[a] | | | |
|---|---|---|---|---|---|---|
| Exposure | | | 0.25 μM | 0.25 μM | 2.0 μM | 2.0 μM |
| (h) | (h) | Size[b] | high | low | high | low |
| 24 | 0 | | 1.11 ± 0.10 | 1.48 ± 0.19* | 1.77 ± 0.28* | 1.99 ± 0.32* |
| 48 | 0 | | 1.38 ± 0.09* | 1.47 ± 0.51 | 1.32 ± 0.10* | 1.12 ± 0.05* |
| 48 | 24 | | 1.68 ± 0.07* | 1.61 ± 0.16* | 2.22 ± 0.09* | 2.24 ± 0.31* |
| 48 | 72 | | 1.06 ± 0.15 | 1.12 ± 0.23 | 0.42 ± 0.11* | 1.13 ± 0.32 |

*$P < 0.05$ (*t*-test) relative to control
[a] Data (mean values relative to untreated control ± SEM) derived from 4 independent experiments
[b] DNA fragment size of 1–50 kb (low) and 50–1,000 kb (high)

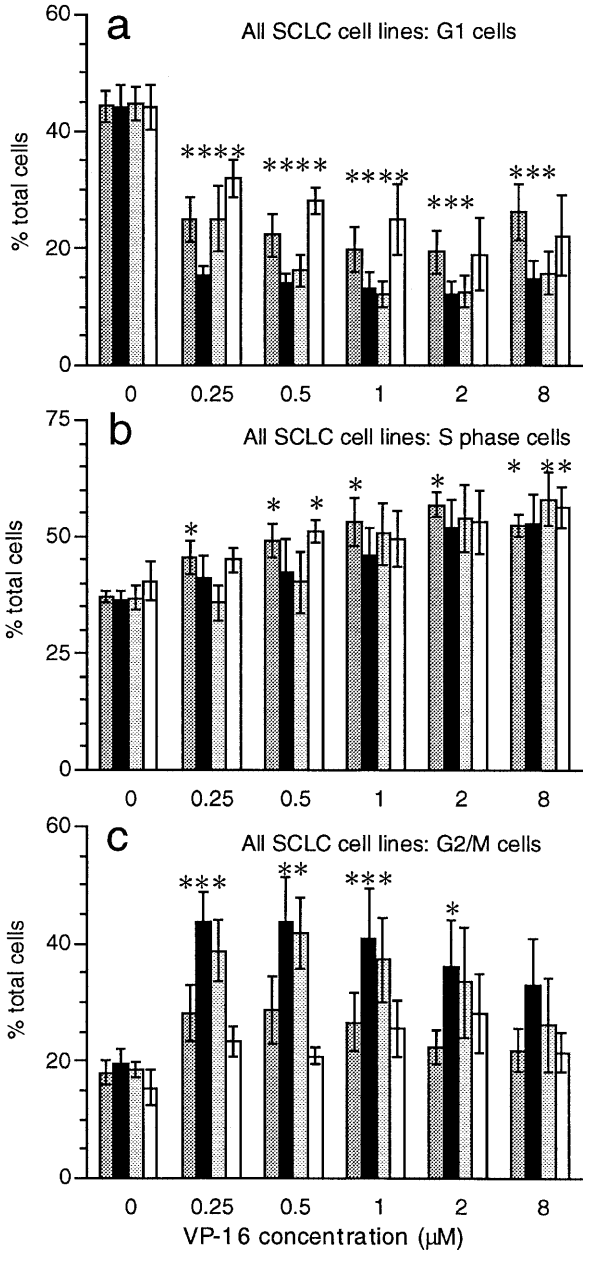


**Fig. 1a–c** VP-16-induced cell cycle perturbations in a panel of seven small-cell lung carcinoma (SCLC) cell lines. The graphs show combined data (means ± SE) for NCI-H69, COR-L32B, COR-L51B, COR-L96C, COR-L103, COR-L266B and COR-L279 cells. **a** G1 cells; **b** S phase cells; **c** G2/M cells. Data derived from 3–5 experiments. *Columns* represent treatment conditions: VP-16 × 24 h (*dark grey*); VP-16 × 48 h (*closed*); VP-16 × 48 h followed by 24 h recovery in drug-free medium (*light grey*) or 48 h followed by 72 h recovery in drug-free medium (*light grey*). *Asterisk* indicates that the difference between the value and paired control is significant at $P \leq 0.05$ (*t*-test)

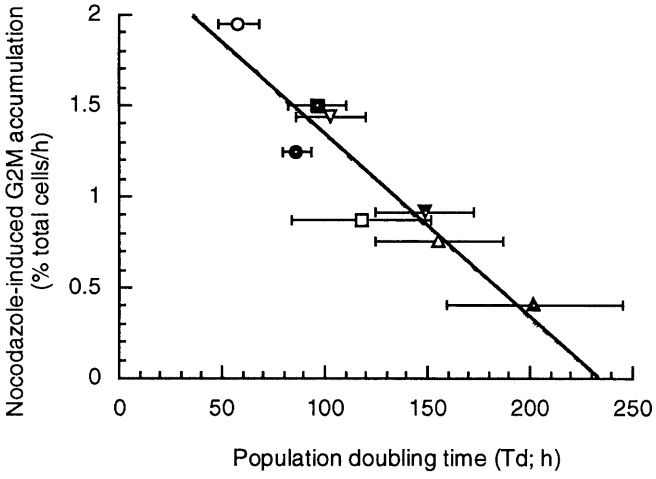


**Fig. 2** Rate of entry of cells into G2/M correlates with gross population doubling time. Initial rate (0–24 h) of accumulation of cells in G2/M calculated for nocodazole-treated cultures. Data (± SE) are derived from 3–5 experiments. Symbols: NCI-H69 (○), COR-L32B (Δ), COR-L47F (▽), (COR-L51B (□), COR-L88B (◇), COR-L96C (●), COR-L103 (▲), COR-L266B (▼), COR-L279 (■)

treatment at 48 h results in a fall in the number of cells retained in G2/M, this recovery or perhaps phase-specific cell loss being particularly evident at the lower VP-16 concentrations. However, the percentage of cells in G2/M does not fall below control values. The frequency of cells in G1 increases during the recovery phase, consistent with mitotic traverse, and is again most evident in the lower concentration range. The S phase fractions during culture recovery show no significant differences from controls at low doses, while 8 μM VP-16 results in elevated and persistent trapping. The data suggest an ongoing S phase slowing, initiated during the first 24-h exposure, that becomes limiting for G2 entry at doses > 0.5 μM and that cells can recover from this effect on DNA replication upon drug release.

## Stathmokinetic analyses of cycle arrest

To assess the relationship between doubling time and the potential for the capture of cells in G2/M, we used nocodazole to block cell division at mitosis (Fig. 2; [13]). The variation in the mitotic (M) population can be monitored by 90 light scatter changes of nuclei [5]. G2/M accumulation over the first 24 h of nocodazole exposure was inversely correlated with population doubling time, with a linear relationship (linear regression $r = 0.928$; Spearman correlation for seven pairs observed $P = 0.014$). These data suggest that the SCLC cell lines show similar intrinsic sensitivity to nocodazole blockade. Using this stathmokinetic approach we investigated the expression of the S phase delay for the group of SCLC cell lines. Figure 3a–c shows the group responses of the cell lines to VP-16 in the presence of the mitotic inhibitor nocodazole. It is clear that over the whole dose concentration range the SCLC group cells can exit G1 essentially unhindered, with approximately 50% of cells being lost from G1 during the first 24-h exposure period. The low-dose-induced reduction in the number of cells reaching G2/M suggests a significant pre-G2/M delay effective during the first 24 h of drug exposure and complete at concentrations of > 2 μM VP-16. The origin

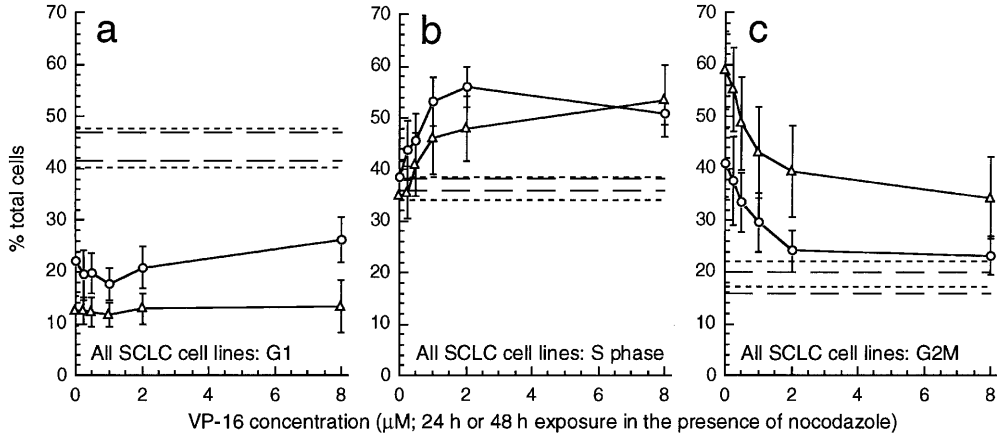


**Fig. 3a–c** VP-16-induced cell cycle perturbations in a panel of seven SCLC cell lines measured in the presence of the mitotic spindle inhibitor nocodazole. The graphs show combined data (means ± SE) for NCI-H69, COR-L32B, COR-L51B, COR-L96C, COR-L103, COR-L266B and COR-L279 cells. **a** G1 cells; **b** S phase cells; **c** G2/M cells. Data derived from 3–5 experiments. Symbols: VP-16 + nocodazole × 24 h (○), VP-16 + nocodazole × 48 h (Δ). The *dashed* and *dotted* lines represent the values (upper and lower limits for the SEM value) for parallel untreated controls evaluated at 24 h or 48 h incubation, respectively

of this effect is a clear dose-dependent accumulation of cells in S phase, becoming saturated at 2 μM VP-16. The reduction in the S phase fraction between 24 and 48 h exposure suggests that many cells are slowed in the S phase rather than trapped. Although the SCLC group shows a clear trend, the error bars suggest considerable inter-cell line variation.

## Early cell cycle arrest and induced DNA fragmentation

Figure 4a shows the initial pre-G2/M cell cycle arrest/delay induced in individual SCLC cell lines by a 24-h exposure to the non-saturating concentration of 0.25 μM VP-16 in the presence of nocodazole. The G2/M entry values are expressed as a percentage of the maximum possible, determined in parallel control cultures treated with nocodazole alone. Cell lines were ranked in terms of increasing expression of the pre-G2/M arrest/delay (i.e. decreasing G2/M entry; Fig. 4a, columns left to right). The results show a wide spread in the expression of pre-G2/M delay from the relatively resistant NCI-H69 cell line to the sensitive COR-51B, COR-L279 and COR-L103 cell lines, in which delay appeared to be maximal.

We investigated whether the extent of DNA fragmentation, using a saturation concentration for the expression of S phase delay to maximise signal/noise ratio, correlated with the rankings for pre-G2/M delay potential. The results shown in Fig. 4b suggest that there is no correlation between the low levels of DNA fragmentation observed and the expression of the pre-G2/M arrest in the SCLC cell line panel. A parallel control

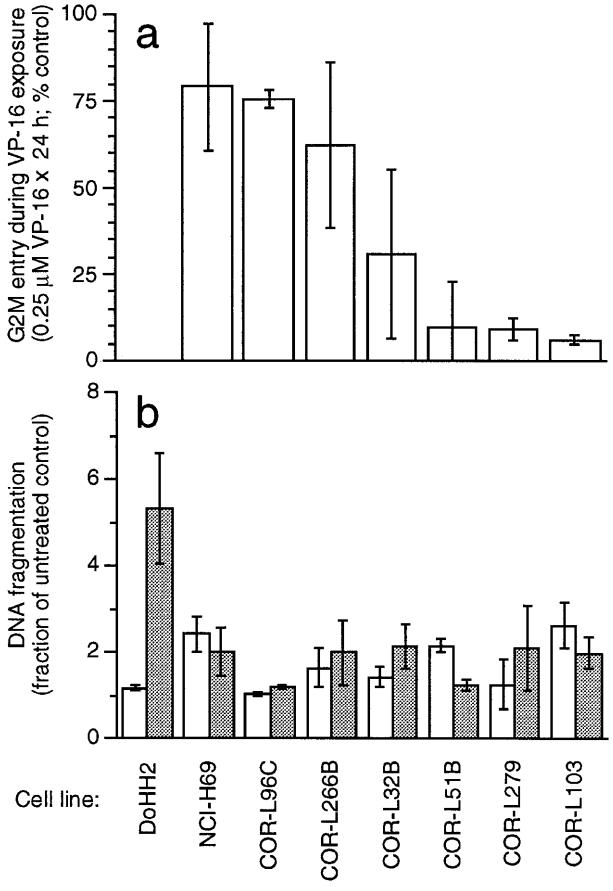


**Fig. 4a, b** Initial pre-G2/M cell cycle arrest/delay and DNA fragmentation induced in SCLC cell lines by a 24-h exposure to 0.25 μM VP-16. **a** G2/M arrest of SCLC cells, as a percentage of the maximum possible as determined in parallel control cultures treated with nocodazole. Cell lines have been ranked in terms of increasing expression of a pre-G2/M arrest/delay. **b** Drug-induced (24 h exposure to 2 μM VP-16) DNA fragmentation in SCLC cultures measured using field inversion gel electrophoresis monitoring of the generation of low (5–50 kb, *shaded columns*) or high (50–700 kb, *open columns*) molecular size DNA fragments. DoHH2 cells were used as positive apoptotic controls generating a more than fivefold increase in DNA fragments in the low molecular weight range alone. Data are means (± SEM) of values from 3–5 experiments

culture of p53 wild type DoHH2 cells was used as a positive apoptotic control generating a more than five-fold increase in DNA fragments, preferentially in the low molecular weight range. The results suggest that the SCLC cell lines are relatively resistant to the early and complete execution of apoptosis. Parallel measurements of apoptotic cell frequency (propidium iodide versus Annexin V binding; terminal deoxytransferase end-labelling of DNA fragments) also indicated muted apoptotic responses in the SCLC cell lines (P.J. Smith, unpublished data).

### Cell cycle arrest and recovery potential of NCI-H69 and COR-L279 cells

To investigate the consequences of these differences in initial arrest potential we selected the NCI-H69 (low S phase arrest potential) and the COR-L279 (high S phase arrest potential) for further study. Both lines show homozygous *TP53* gene abnormalities. They also show the largest difference in arrest potential balanced against the smallest difference in population doubling time. We note that the COR-L279 line appears to be at least twice as sensitive to VP-16 as NCI-H69 for more-extended exposure conditions (Table 1). Figure 5a–c shows the cell cycle changes observed at 48 h and their subsequent resolution over a 24-h recovery period. NCI-H69 cells show a clear ability to reduce the fraction of cells accumulated in G2/M during the recovery period, with loss of recovery at ≥2 μM VP-16. This loss of accumulated NCI-H69 cells in G2/M is accompanied by an increase in G1 fraction, but not to the extent expected of a one to two cell division rate, suggesting that some cells may be lost to limited fragmentation events during this process.

The characteristics of the COR-L279 culture are clearly different in that G2/M accumulation at 48 h VP-16 exposure occurs only at very low doses and is maximal at 0.25 μM. At concentrations between 0.5 and 2 μM and beyond there is evidence of a dose-dependent decrease in G2/M entry commensurate with the appearance of cells delayed in S phase. Unlike NCI-H69, the COR-L279 cell line shows little or no change in G2/M fraction during the 24-h post-treatment incubation period (compare values for G2/M fractions at 0.5 μM VP16; Fig. 5c). Importantly, the clear differences between the cell lines is also evident at the high concentration of 8 μM VP-16, suggesting that high levels of supra-toxic damage or imposed arrest per se in the NCI-H69 culture cannot generate a COR-L279-like response.

Despite the apparent lack of recovery of the G2/M fraction in COR-L279, at the lower drug concentrations there is a regaining of cells in G1 (Fig. 5a), suggesting that some cells can escape G2/M arrest but that the late cell cycle fraction continues to be supplied from S phase during the recovery process. Figure 5a also shows that the emptying of G1 becomes limiting at ≥2 μM VP-16 after 48 h exposure, and that NCI-H69 and COR-L279 differ in the absolute residual fraction of cells remaining in G1. This residual fraction could comprise drug-trapped, $G_0$, slow cycling or moribund cells.

## Discussion

As a group the SCLC cell line panel showed early (within 24 h) and progressive responses to VP-16 in terms of cell cycle perturbations. The patterns of arrest were consistent with the p53 mutant genotypes in the absence of G1/S arrest, the major delays occurring in the first cycle. The main finding is that cell cycle responses of the different SCLC cell lines to VP-16 appear to be underpinned by wide variation in the expression of S phase delay. The range of sensitivities to this pre-G2 delay arrest is not attributable to differences in population doubling rate per se. The relatively low level and protracted nature of apoptosis in the SCLC cultures did not appear to confound the analyses of drug-induced arrest

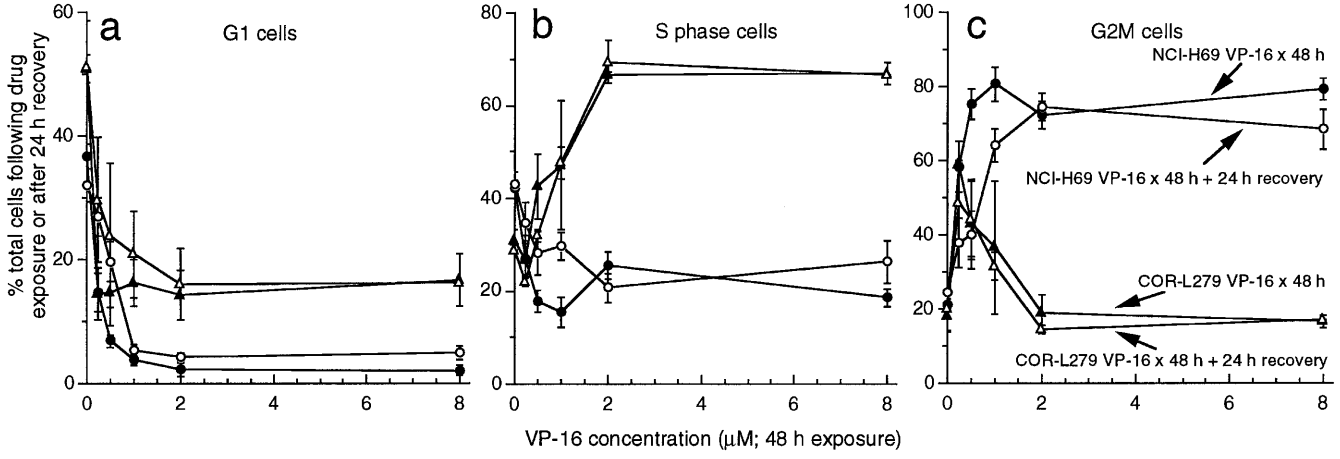


**Fig. 5a–c** Induction and resolution of VP-16-induced cell cycle perturbations in NCI-H69 and COR-L279 cell. Changes in G1 (**a**), S phase (**b**) and G2/M (**c**) cells. Data are mean values (±SE) for 3–5 experiments. Symbols: NCI-H69, (●, ○) and COR-L279 (▲, Δ) treated with VP-16×48 h (*closed symbols*) or VP-16×48 h followed by 24 h recovery in drug-free medium (*open symbols*)

during the first exposure 48-h period and did not correlate with the severity of the pre-G2 delay/arrest. The MTT assay data were used to confirm the likely biologically relevant drug concentration range and not to seek concordance between these essentially different assays.

The comparison of the NCI-H69 and COR-L279 cell lines in which there was no evidence of an inhibition of S phase entry, consistent with their *TP53* homozygous mutation, reveals a large difference in the impact of VP-16 on S phase traverse rather than G2 arrest potential. Importantly, we also demonstrate that this difference cannot be compensated for by drug concentration changes alone. We show clear evidence that at least for the NCI-H69 versus COR-L279 comparison the attainment of maximal G2/M delay/arrest is determined by the threshold sensitivity for S phase delay. On the other hand all of the cell lines show a saturation of arrest potential at drug doses ≥2 μM VP-16 (exposure dose value; ED value of concentration of drug×time ≥48 μM.h). This would specify a minimal in vitro treatment condition that induces cell cycle arrest despite the inherent heterogeneity in the response patterns of SCLC cells. Differential sensitivity could be described by comparing the minimal ED values required to achieve a state in which recovery from a G2/M checkpoint arrest does not occur within a defined period (e.g. 24 h), for whatever reason. Abstracting these values from Fig. 5c indicates that the NCI-H69 and COR-L279 cell lines show a fourfold difference in ED values of ≤ 24 μM.h and 96 μM.h, respectively. The ED values are consistent with a recent multiparameter flow cytometric analysis of different M1 myeloid leukemia clones that lack p53, express mutant p53 constitutively or which express temperature sensitive wild type p53 [24], in that lower VP-16 exposures (ED 20.4 μM.h) were associated with a block in the G2. The current findings demonstrate the complexity of the schedule-dependent responses of p53 mutant SCLC cells to VP-16 and would be consistent with the variable expression of S phase checkpoint activation.

It is difficult to compare drug response characteristics for in vitro experiments with those for patient studies, because of the assumptions that have to be made to account for the intrinsic sensitivity of the target cells, effects of drug metabolism and drug bioavailability. Plasma concentration versus time curves (AUC) following an intravenous dose of VP-16 can also exhibit intra- and inter-subject variability [23]. However, over a dose range of 100–600 mg/m$^{-2}$, AUC and the maximum plasma concentration (*C*max) values increase linearly with dose. The disposition of VP-16 is best described as a biphasic process with a distribution half-life of about 1.5 h and terminal elimination half-life ranging from 4 to 11 h. In terms of VP-16 steady-state plasma concentrations in SCLC patients, Porter et al. [23] have used target concentrations of 1.7 and 3.4 μM V-16 (delivered as etoposide phosphate) for continuous 5-day infusion schedules in a therapeutic drug monitoring study. In a different study investigating the effect of organ function on total and free VP-16 pharmacokinetics and haematological toxicity, VP-16 AUC calculations for single-agent intravenous administration over 5 or 8 days indicated a fraction for free drug/total drug of 0.04 [12]. Calculating the potential plasma concentration ranges for free (0.16–0.25 μM) and total (4–6.4 μM) drug yields a free drug ED value of 30 μM.h. This value corresponds closely with the identification in the present study of ED values of 24 μM.h (i.e. 0.5 μM×48 h) as the minimal condition to generate maximal G2/M arrest but also allow for cell recovery. However, the results of a randomised SCLC trial investigating the activity and toxicity of continuous infusion etoposide phosphate revealed low activity at 1 μg/ml for a 15-day arm (calculated ED for free VP-16 equivalent to 24.5 μM.h), suggesting that this regime is below the therapeutic window [11]. This agrees well with the results of the current SCLC group analyses that suggest that an ED ≥48 μM.h defines a minimal in vitro treatment condition that induces overall cell cycle arrest and in which recovery potential is simultaneously compromised.

The implication of our findings is that variation in sensitivity to S phase arrest, clearly demonstrable within the current panel of recently derived SCLC cell lines, occurs within ED ranges for which comparable in vivo regimens may be below the therapeutic window [11]. We suggest that such variation will impact upon individual tumour responsiveness and thereby contribute to resistance and treatment failure. We further suggest that this variable expression of stress-induced p53-independent S phase arrest, may reflect differential expression of S phase checkpoint(s) and could be a therapeutic target. Cells escaping VP-16-induced S phase arrest would offer persistent DNA replication as a functional target. The feasibility of appropriate drug combinations to target this evasion route is suggested by the report that the S phase targeting DNA topoisomerase I inhibitor topotecan, which requires active DNA replication for the induction of cytotoxic DNA damage, has modest antitumour activity in SCLC patients refractory to etoposide and cisplatin [22]. Such combination studies are currently under investigation in vitro.

## References


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